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14. ABSTRACT: We have made very significant progress towards the completion of the goals proposed in this award. In the first of the two Aims, we proposed to generate cell lines in which we stably suppressed XIAP using lentiviral-based RNA interference, and subsequently to constitute XIAP expression using mutants which are incapable of suppressing caspases. While we have achieved these goals using PC-3 cells, we have encountered some issues of non-specificity when these lines are examined in xenograft models, and we are in the process of troubleshooting and examining alternative approaches to address these questions. In the second Aim, we proposed to examine XIAP expression in the TRAMP and Pten conditional transgenic murine models of prostate cancer. We have completed these studies in the TRAMP model, and found that surprisingly, there is little difference in the rates of tumor onset and the survival time of Xiap null mice, compared to littermate controls groups. Interestingly, there is a slight trend towards Xiap-deficient animals being more susceptible to tumors, which may have significant implications for the use of XIAP antagonists as anti-cancer agents.

15. SUBJECT TERMS
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Introduction

Expression of the X-linked inhibitor of apoptosis (XIAP) protein has been shown to be elevated in a growing number of malignancies, most notably prostate cancer, and this has led intense interest in XIAP as a potential molecular target. While it is generally thought that XIAP supports oncogenesis by virtue of its apoptosis suppressing effects, the central hypothesis of our research is that this property of XIAP is not its primary role. We proposed a series of experiments to establish which functions of XIAP, whether or not they are related to apoptotic inhibition, participate in the tumor-supporting properties of this molecule.

Body

We have made significant progress towards completing the tasks outlined in the original Statement of Work, as summarized below:

SPECIFIC AIM 1. To dissect and evaluate the contributions of the distinct caspase inhibitory and signaling functions of XIAP in the development of prostate cancer.

In this aim we proposed to use lentiviral-mediated RNA interference to generate clones of prostate cancer lines lacking XIAP. We proposed to subsequently reintroduce mutants of XIAP variously lacking either the caspase-inhibitory or E3 ubiquitin ligase activity of XIAP. We have made very significant progress in this area. As outlined in our previous report, we have concentrated on the PC-3 cell as a model to test our reagents and the experimental model, and we have effectively ablated XIAP with these mutants. We have now had the opportunity to extensively analyze these lines (Figure 1), as well as 'rescue' lines in which the XIAP mutants have been reintroduced, in xenografts. As can be seen in Figure 1, while there is a modest suppression of tumorigenicity in cells lacking XIAP, the suppression observed is only slightly that observed in control lines transduced with an irrelevant RNAi cassette. Both groups of transduced lines appear to form tumors at a time following injection which is significantly delayed relatively to parental, untransduced cells, and this gives us cause for concern, since it raises the possibility that an immune response is being generated against these lentivirally modified tumor lines which would hamper subsequent analysis of 'rescue' lines and prevent us from establishing an experimental window to conclusively gauge our results. Additionally, the experimental variation in these lines is very significant. One possible explanation for this would be a relatively non-specific interferon response, perhaps initiated by the expression of the dsRNA hairpin. While we cannot conclusively exclude this possibility, we have tested for the expression of interferon-responsive genes by real-time quantitivative RT-PCR, and have not observed any significant differences in the various cell lines. However, we note now that relatively few examples have been described in the literature of this approach being taken, although at the time of proposing and first performing our studies the technique of RNA interference was so novel that no data had been presented for or against it being successful in xenograft studies.

To address these issues, we are taking the following approaches:

a) To test the possibility that the siRNA target sequence in XIAP is, for unknown reasons, inducing an anti-tumor, off-target effect, we are designing and testing additional XIAP-specific sequences, and will proceed to examine effective siRNAs in xenograft studies.

b) To examine the possibility that the parental PC-3 cell line is particularly susceptible to the effects of lentiviral transduction, and if for off-target reasons we determine that lentivirally transduced derivatives of this cell line cannot be used for xenograft studies, we will extend

perform comparative studies in the three additional cell lines proposed in our original report: DU145 (which, like PC-3, is androgen-independent), LNCaP and MDA PCa2b (both androgen-dependent).

b) If, after performing the additional experiments described above in (a) and (b), we conclude that we are unable to utilize our lentiviral system to specifically suppress XIAP and obtain statistically significant xenograft data, we will request the antisense targeting vector used to suppress XIAP in numerous tumor lines (described by McManus *et al*, *Oncogene* 23:8105-8117, 2004). Stable suppression of XIAP using this vector has been reported to exhibit dramatically impaired growth in xenograft studies. For calibration and trouble-shooting purposes, we will also request from the authors of this study the breast cancer line, MDA-MB-231, because this cell line was described in most detail in their study. However, we will use the antisense vector to stably suppress XIAP in PC-3 cells , and will use PC-3 derivatives generated in this manner in a parallel xenograft study along with our existing lines suppressed through a lentiviral approach.

SPECIFIC AIM 2. To validate XIAP as a novel target for the treatment of prostate cancer, using established transgenic and conditionally targeted murine models.

2.1 Analysis of the rate of tumor progression in *Xiap*-deficient mice.

a) Examination of Xiap expression profiles in TRAMP and Pten conditionally deficient mice. In this sub-aim, we proposed firstly to confirm a previous report that Xiap expression is enhanced in the TRAMP, and ultimately Pten-conditional, murine models of prostate cancer. As described in our previous annual report, for the TRAMP model this has been completed.

b) Evaluation of the contribution to tumorigenesis of XIAP using *Xiap*-targeted mice. The second goal of this sub-aim was to breed our Xiap-deficient mice to TRAMP (and ultimately Pten-conditional) mice, and examine tumor burden. For the case of the TRAMP model, this section of work is now complete. As shown in Figure 2, we have found no statistically significant differences between Xiap-deficient mice and littermate controls, in terms of tumor onset, animal survival and general pathology. It is possible that the TRAMP system may be too rapid and aggressive a model, and the Pten model may be more sensitive in this regard. We are still in the process of obtaining these mice, or from two local investigators who are breeding the prostate-specific Cre transgenic mouse and Pten floxed mouse. These studies are therefore in progress, and in the future if funding permits we would consider adding an an additional arm to these murine studies using a chemotherapeutic drug, e.g. taxotere, to determine whether there are any combined effects of XIAP deficiency and therapy.

2.2 Use of murine models of prostate cancer to evaluate the effectiveness of XIAP-specific antisense strategies.

In this sub-aim, we proposed to use stabilized antisense oligonucleotides designed and validated specifically to target murine Xiap, in a study to examine their effects on tumor development in TRAMP and Pten-conditional murine prostate cancer models. The dosage regimen for this reagent (in parallel with a scrambled control oligonucleotide) is underway in our first round of TRAMP transgenic mice. From our preliminary studies, we have found good evidence that adminstration of Xiap-specific antisense elicits an anti-tumor response. However, we have also observed a number of non-specific effects that are deleterious to the mice using the scrambled non-specific control. We are therefore working with the group providing the

antisense reagents (Aegera, Inc.) to further understand the nature of these non-specific effects. In summary, these studies are in progress.

Figures

Figure 1. Tumor formation of XIAP-deficient prostate carcinoma cells. Tumorigenicity of parental PC3 cells (blue), control shRNA PC3 cells (green) and XIAP shRNA PC3 cells (red) was assessed by injection into immunodeficient mice. Athymic nude mice were subcutaneously injected with 5×10^6 PC3 cells/ injection site. Tumor growth was monitored weekly, and tumor size was estimated based on orthogonal caliper measurements, using $mass = a \times b^2 / 2$, where *a* and *b* are the longest and shortest measurements, respectively. Mice displaying any apparent distress were humanely euthanized.

Figure 2. Tumor formation in XIAP-deficient TRAMP mice. XIAP-deficient TRAMP mice (solid line, n=13) and wild type TRAMP littermates (dotted line, n=11) were monitored by weekly abdominal palpation and biweekly MRI. Moribund mice were humanly euthanized. (A) Tumor onset of palpable abdominal tumor. (B) Overall survival.

Figure 1

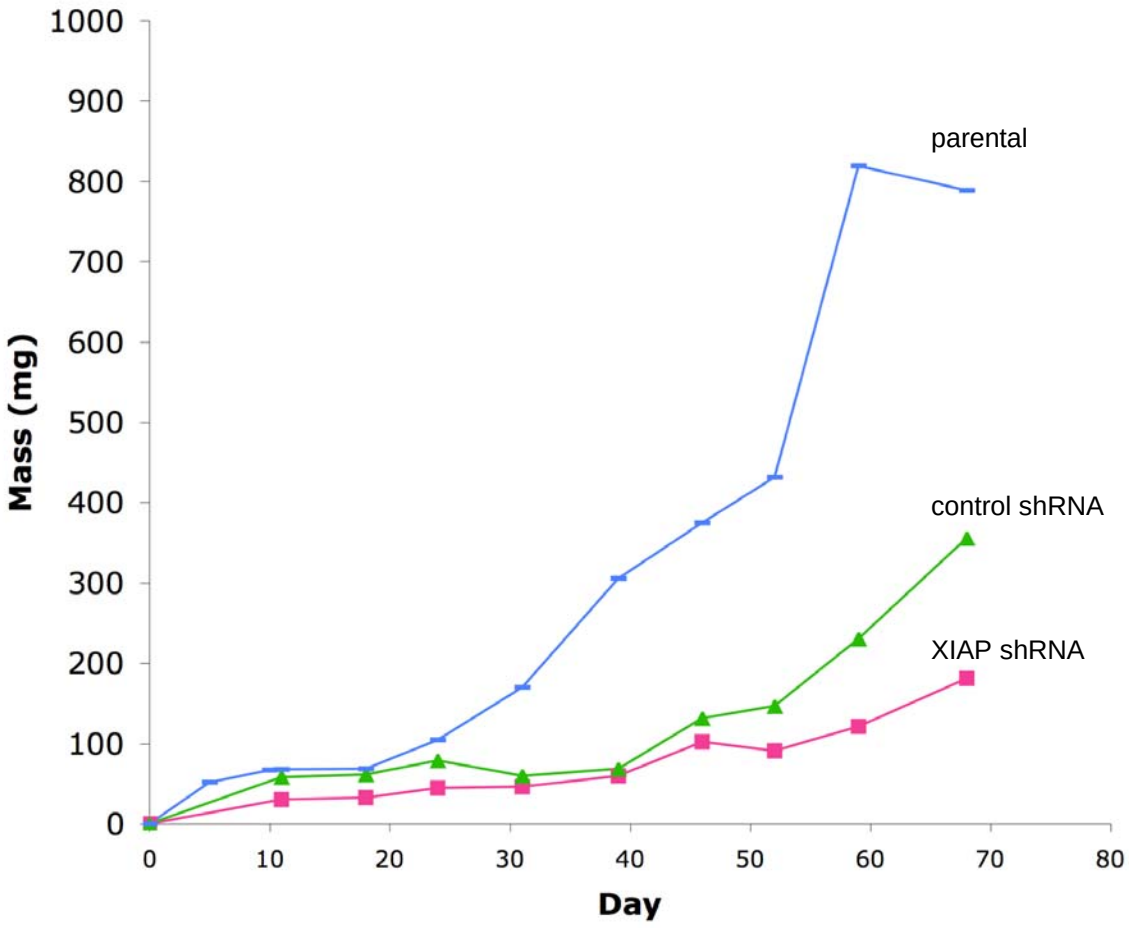
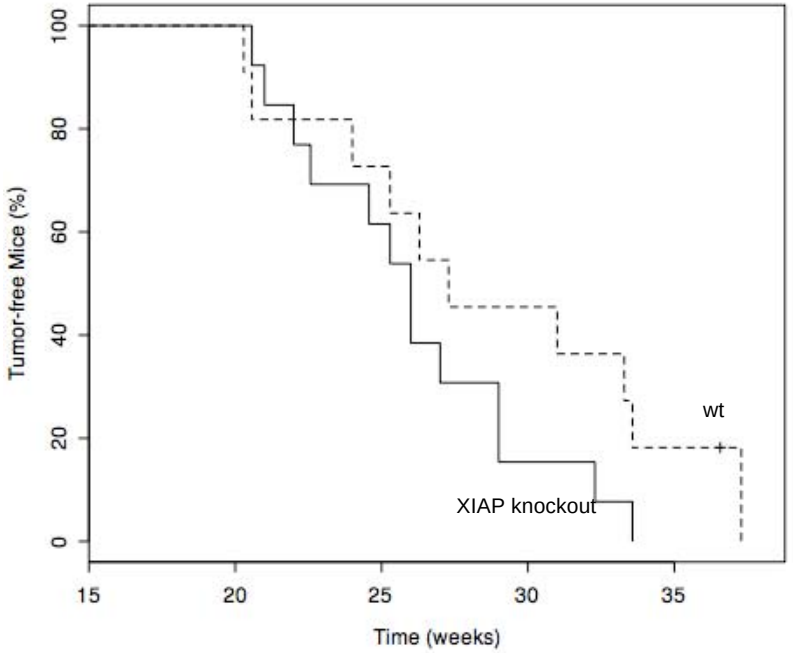
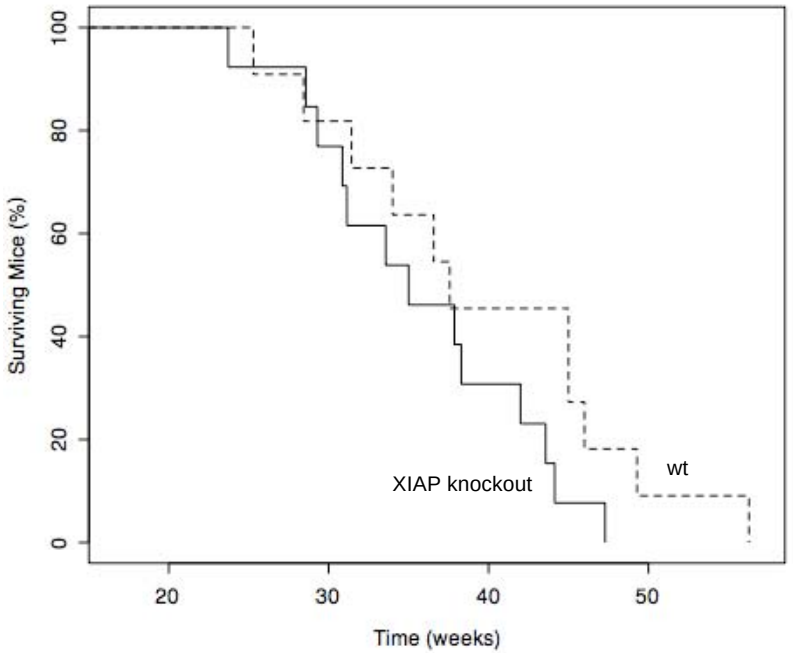

1000
900
800
700
600
500
400
300
200
100
0
Mass (mg)
0
10
20
30
40
50
60
70
80
Day
parental
control shRNA
XIAP shRNA

Figure 2

(a)


Tumor-free Mice (%)
0
20
40
60
80
100
15
20
25
30
35
Time (weeks)
wt
XIAP knockout

(b)


Surviving Mice (%)
0
20
40
60
80
100
20
30
40
50
Time (weeks)
wt
XIAP knockout

Key Research Accomplishments

- Generated PC-3 prostate cancer cell line derivatives lacking XIAP, as well as control lines with irrelevant RNAi target sequences.
- Examined the tumor growth effects of these lines in xenografts
- Bred XIAP-deficient mice to TRAMP mice.
- Compared the onset and mortality of TRAMP mice in a wild type and XIAP-null background.

Reportable Outcome

The following manuscripts have been published in this reporting year, and cite this grant:

Mufti, A.R., Burstein, E., Csomos, R.A., Graf, P.C.F., Wilkinson, J.C., Dick, R.D., Challa., M., Son, J.-K., Bratton, S.B., Su, G.L., Brewer, G.J., Jakob., U. and **Duckett, C.S.** XIAP is a copper binding protein deregulated in Wilson's Disease and other copper toxicosis disorders. *Mol. Cell* **21**:775-785 (2006).

Conclusions

While considerable progress has been made in this reporting year, we are currently addressing a technical issue relating to the use of lentivirally transduced prostate cancer lines in xenograft studies. We are in the process of determining whether the lentiviral approach itself is causing generalized anti-tumor response in the mouse, or whether the siRNA target sequences are inducing off-target effects. Secondly, we have made good progress with hopefully reportable outcomes in the evaluation of the TRAMP murine model of prostate cancer in Xiap-null animals. In summary, we believe our studies are continuing to develop, and expect to be able to solve some technical issues that have arisen.

Appendix

Mufti, A.R., Burstein, E., Csomos, R.A., Graf, P.C.F., Wilkinson, J.C., Dick, R.D., Challa., M., Son, J.-K., Bratton, S.B., Su, G.L., Brewer, G.J., Jakob., U. and **Duckett, C.S.** XIAP is a copper binding protein deregulated in Wilson's Disease and other copper toxicosis disorders. *Mol. Cell* **21**:775-785 (2006).

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XIAP Is a Copper Binding Protein Deregulated in Wilson's Disease and Other Copper Toxicosis Disorders

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Summary

X-linked inhibitor of apoptosis (XIAP), known primarily for its caspase inhibitory properties, has recently been shown to interact with and regulate the levels of COMMD1, a protein associated with a form of canine copper toxicosis. Here, we describe a role for XIAP in copper metabolism. We find that XIAP levels are greatly reduced by intracellular copper accumulation in Wilson's disease and other copper toxicosis disorders and in cells cultured under high copper conditions. Elevated copper levels result in a profound, reversible conformational change in XIAP due to the direct binding of copper to XIAP, which accelerates its degradation and significantly decreases its ability to inhibit caspase-3. This results in a lowering of the apoptotic threshold, sensitizing the cell to apoptosis. These data provide an unsuspected link between copper homeostasis and the regulation of cell death through XIAP and may contribute to the pathophysiology of copper toxicosis disorders.

Introduction

Copper, an essential trace metal, is a catalytic cofactor for enzymes that play critical roles in a number of biological processes, including oxidative phosphorylation (e.g., cytochrome c oxidase) (Hamza and Gitlin, 2002) and oxidative stress protection (e.g., superoxide dismutase) (Torres et al., 2001). As copper is potentially toxic to cells, an intricate mechanism for handling intracellular copper ions has evolved, and free copper in the cell is almost undetectable (Rae et al., 1999).

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Mutations in genes involved in copper homeostasis are responsible for disorders of copper metabolism in humans (Mercer, 2001). The best-described human copper toxicosis disorder, Wilson's disease (Llanos and Mercer, 2002), results from mutations in *ATP7B*, a gene encoding a P type ATPase. It is characterized by the pathologic accumulation of copper in the liver and brain (Brewer, 2000; Gitlin, 2003). A number of similar syndromes that are not due to mutations in *ATP7B* are collectively referred to as non-Wilsonian copper toxicosis. Recently, a mutation in the previously undescribed gene *COMMD1* (also known as *MURR1*; [Burstein et al., 2004]) was shown to be responsible for an autosomal recessive form of a non-Wilsonian copper toxicosis disorder affecting Bedlington terriers (Klomp et al., 2003; van De Sluis et al., 2002). COMMD1 has recently been reported to interact with ATP7B (Tao et al., 2003), although the functional role of this interaction remains to be elucidated.

We independently identified COMMD1 as a direct binding partner of the prosurvival protein XIAP (Burstein et al., 2004). XIAP is best known as a conserved metazoan protein (Duckett et al., 1996; Liston et al., 1996; Uren et al., 1996) that can suppress apoptosis by directly binding to and inhibiting the catalytic activity of several caspases (Chai et al., 2001; Deveraux and Reed, 1999; Riedl et al., 2001; Riedl and Shi, 2004; Shiozaki et al., 2003). Additionally, it has also been shown that XIAP regulates cellular levels of COMMD1 by functioning as an E3 ubiquitin ligase for this protein and promoting its proteasomal degradation (Burstein et al., 2004; Vaux and Silke, 2005; Yang et al., 2000). Consistent with this, changes in XIAP expression result in alterations of intracellular copper levels, and *Xiap*-deficient mice have copper deficiency in conjunction with increased COMMD1 levels (Burstein et al., 2004).

The identification of a role for XIAP in copper homeostasis prompted us to investigate the effects of copper on XIAP expression and function. In the course of these studies, we found that in situations associated with elevated copper, XIAP protein levels are greatly reduced and, when detectable, exhibit an altered electrophoretic mobility, which we show is indicative of a major conformational change in its structure. These alterations in XIAP levels and conformation are independent of COMMD1, because they are detected not only in affected Bedlington terriers but also in other canine and murine copper toxicosis diseases, as well as in biopsy material from patients affected with Wilson's disease. We report that the altered electrophoretic mobility of XIAP is due to a reversible conformational change in the protein induced by direct binding of copper to cysteine residues within the BIR and RING domains of XIAP. The copper bound form of XIAP in cells is significantly less stable than native XIAP and is greatly impaired in its ability to inhibit caspase-3, and cells expressing it are more susceptible to apoptosis. These findings suggest a model in which changes in intracellular copper levels can affect the apoptotic threshold by determining the level and activity of XIAP and provide an additional

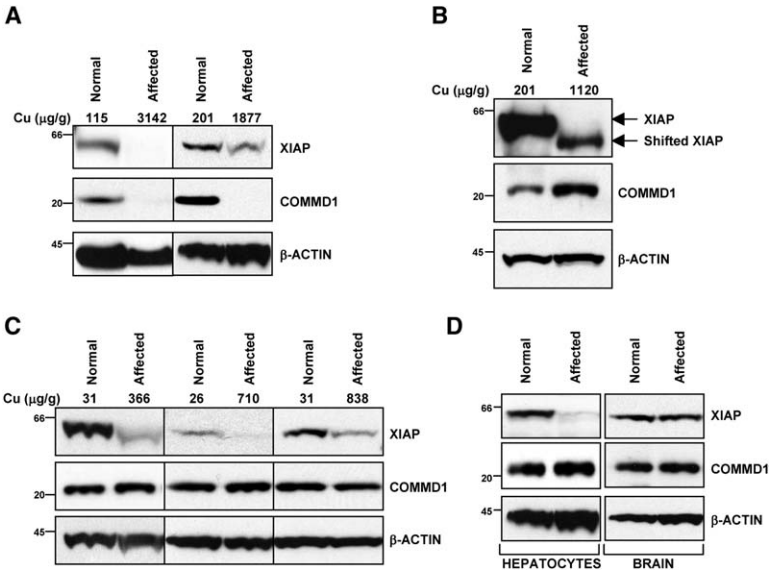

Figure 1. Altered Expression of XIAP in Copper Toxicosis Disorders

(A) Immunoblot analysis of liver tissue revealed decreased levels of XIAP in Bedlington terriers with inherited copper toxicosis (affected), compared to control animals (normal).

(B) Altered mobility of XIAP in a cocker spaniel with hepatic copper accumulation due to cholestasis. Hepatic tissues from an unaffected Bedlington terrier and an affected cocker spaniel were lysed and subsequently probed for XIAP, COMMD1, and β-actin.

(C) Decreased hepatic levels of XIAP in patients affected with Wilson's disease. Hepatic tissues from patients with Wilson's disease and normal controls were lysed and subsequently probed for XIAP, COMMD1, and β-actin.

(D) Decreased XIAP levels in hepatocytes, but not in brain tissue, from *Atp7b* mutant mice, the murine model of Wilson's disease. Purified hepatocytes and brain tissue from mutant or control animals were isolated, lysed, and subsequently probed for XIAP, COMMD1, and β-actin.

pathophysiological mechanism for the cellular damage observed in Wilson's disease and other copper toxicosis syndromes.

Results

Impaired Expression and Altered Mobility of XIAP in Disease States Associated with Copper Toxicosis

Genetic studies of Bedlington terriers affected with a hereditary, non-Wilsonian copper toxicosis disorder led to the discovery of the *COMMD1/MURR1* gene (van De Sluis et al., 2002). Because we had previously identified COMMD1 as an XIAP-interacting factor (Burstein et al., 2004), we used immunoblotting to compare the levels of canine XIAP protein in liver tissue samples from Bedlington terriers affected with copper toxicosis and normal control dogs. Interestingly, liver tissue from affected dogs expressed undetectable or greatly reduced XIAP levels compared to controls (Figure 1A). COMMD1 protein was undetectable in affected animals (Figure 1A, middle), confirming that the *COMMD1* locus is indeed targeted in these dogs.

The biopsy data from Bedlington terriers described above suggested that altered expression of XIAP might be due either to the elevated levels of copper in the liver of these animals or to the absence of COMMD1, perhaps through a regulatory feedback process. To discriminate between these two possibilities, we sought to examine pathophysiological situations involving elevations in levels of copper but in which COMMD1 is unaffected. Cholestasis is a condition in which bile excretion from the liver into the digestive tract is blocked, and because bile serves as the main excretory mechanism for copper (Gitlin, 2003), it also results in excessive copper accumulation in the liver. We therefore compared liver tissue from a dog with chronic cholestasis and high hepatic copper levels, but with a wild-type *COMMD1* gene, to a normal, unaffected animal. As shown in Figure 1B, XIAP in the affected animal was not only reduced in level when compared to a normal control but also additionally appeared as a faster-migrating species, under standard reducing and denaturing conditions used for polyacrylamide gel electrophoresis.

The data described above from canine biopsy samples raised the intriguing possibility that XIAP might be affected in human diseases of copper toxicosis. To explore this possibility, pairwise immunoblot analysis was used to compare biopsy samples from patients with Wilson's disease to normal individuals. Similar to the findings from dogs affected with copper toxicosis, liver tissue from patients with Wilson's disease contained lower levels of XIAP protein (Figure 1C), compared to normal, unaffected controls run in parallel. In at least one instance, this reduction was also accompanied by changes in the electrophoretic mobility of the protein (Figure 1C, left), similar to the effects displayed in the canine cholestatic model observed in Figure 1B. Taken together, these data suggested that copper accumulation is associated with reduced XIAP levels, together in some instances with an altered electrophoretic mobility of the protein.

The changes in the mobility and levels of XIAP could result from either direct copper accumulation in the tissue examined or from a generalized alteration of XIAP metabolism secondary to the disease state. To discriminate between these two possibilities, we examined a murine model for Wilson's disease that harbors a spontaneous mutation in the *Atp7b* locus, resulting in the massive accumulation of copper in the liver with only limited changes in brain copper levels (Buiakova et al., 1999; Fuentealba and Aburto, 2003). Similar to the data shown in Figures 1A–1C, hepatocytes from the affected animal contained markedly decreased levels of XIAP, whereas in the same animal, there were no changes in XIAP protein levels in brain tissue (Figure 1D). These findings suggest that the specific effect on XIAP observed in hepatocytes is the direct result of copper accumulation in these cells.

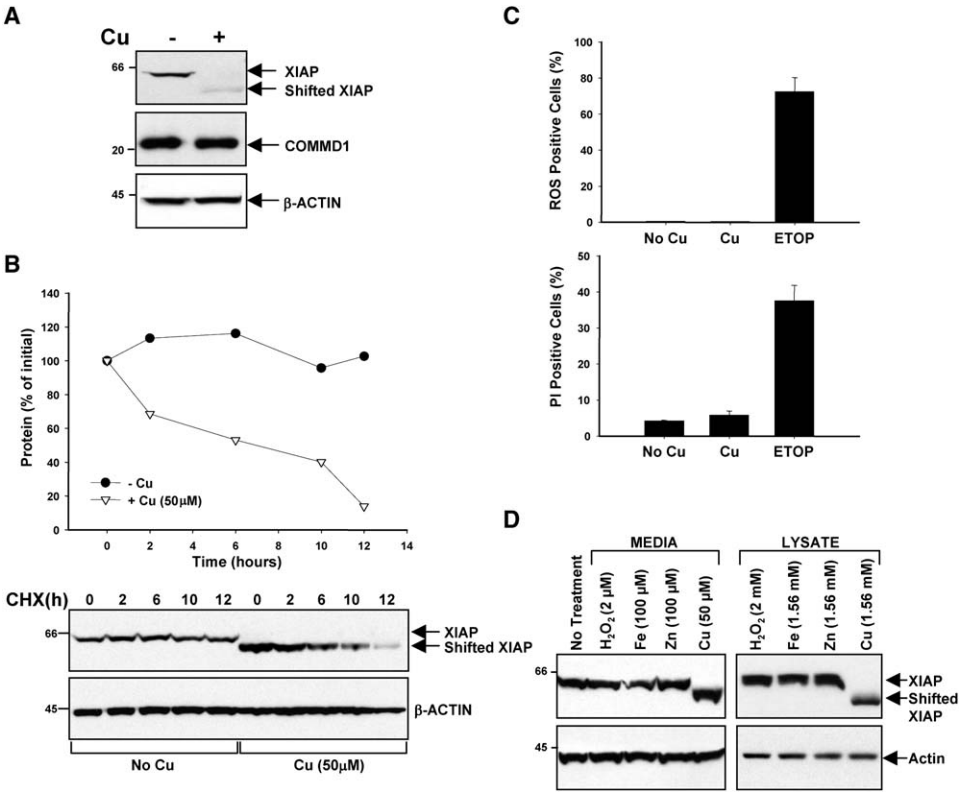

Figure 2. Copper Induces Altered Electrophoretic Mobility and Decreased Half-Life of XIAP

(A) HEK 293 cells were grown in the presence of additional copper sulfate (50 μM). These cells were lysed 48 hr after the addition of copper, and XIAP and β-actin were identified by immunoblotting.

(B) The copper bound form of XIAP has a shorter half-life than native XIAP. HEK 293 cells were grown in the presence or absence of copper sulfate (50 μM) for 48 hr. The cells were then lysed after being incubated with cycloheximide (CHX; 30 μg/ml) as indicated, and XIAP and β-actin were identified by immunoblotting.

(C) The addition of copper sulfate (50 μM) does not result in increased ROS production or cell death. HEK 293 cells were incubated with copper for 48 hr and subsequently probed for the generation of ROS or increased cell death by flow cytometry using CM-H_2DCFDA (1 μM) or propidium iodide, respectively. Etoposide (30 μg/ml) was used as a positive control in both experiments. The error bars represent the standard deviation of three independent samples.

(D) The mobility shift of XIAP is copper specific. HEK 293 cells were grown in the presence of additional copper, iron, zinc, and hydrogen peroxide as indicated. Copper, iron, zinc, and hydrogen peroxide were also added postlysis to untreated HEK 293 cell lysates as indicated. XIAP and β-actin were detected by immunoblotting.

Copper Accumulation Induces Instablility of XIAP Protein

Although the data presented above provided compelling evidence to suggest that XIAP may be altered in expression and mobility in disorders associated with copper toxicosis, these situations do not provide an experimentally tractable system to examine the relationship between XIAP and copper. We therefore tested the possibility that the addition of copper to cells in culture might be able to recapitulate the observations made in animals and patients. As shown in Figure 2A, we could mimic the observations made in biopsy samples by supplementing the media of human embryonic kidney 293 cells with low micromolar concentrations of excess copper, provided in the form of copper sulfate (Figure 2A). Added copper resulted in reduced XIAP levels and also induced a change in the electrophoretic mobility of XIAP resembling those observed in several of the biopsy samples examined in Figure 1. These results were reproduced by using copper chloride as an alternative source of supplemental copper, and similar results were also observed in HepG2 cells and CaCo2 cells (data not shown). The induction of the change in the electrophoretic mobility of XIAP was observed to be time dependent, appearing 24 hr after the addition of copper to culture medium (Figure S1A available in the Supplemental Data with this article online).

To assess whether the reduction in XIAP levels observed reflects changes in XIAP stability in the presence of copper, time decay experiments were performed. HEK 293 cells were cultured in growth media alone or supplemented with copper in order to induce the mobility shift of XIAP, and cycloheximide (CHX) was added to the media to block de novo protein synthesis. XIAP levels were examined by immunoblotting and densitometry after the addition of CHX (Figure 2B). Over a 12 hr period, the levels of XIAP in the control samples were essentially unchanged, indicating a half-life of XIAP significantly greater than 12 hr in the absence of copper. However, XIAP appeared greatly destabilized in the copper-treated cells, which exhibited the electrophoretically altered form of XIAP (Figure 2B). In this case, the half-life of XIAP induced to shift by the addition of copper was ~6 hr (Figure 2B). Quantitative real-time PCR

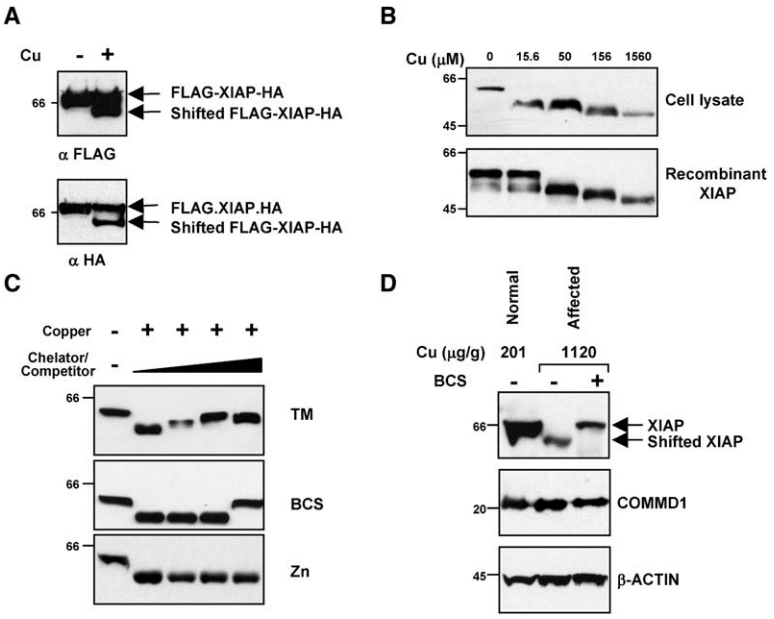

Figure 3. The XIAP Mobility Shift Is Not a Proteolytic Event and Is Reversible

(A) Doubly epitope-tagged XIAP containing an amino-terminal FLAG tag and a carboxy-terminal HA tag was expressed in HEK 293 cells. Copper sulfate (50 μM) was added to the media, and the cells were subsequently lysed. XIAP was detected by immunoblotting using FLAG and HA antibodies as indicated.

(B) The copper-induced mobility shift can be recapitulated in vitro. Increasing amounts of copper were added postlysis to untreated HEK 293 cell lysates and to recombinant XIAP prepared in *E. coli* as indicated. XIAP mobility was determined by immunoblotting.

(C) The copper-mediated mobility shift of XIAP is reversible. The XIAP shift was induced by the addition of copper sulfate (1.56 mM) to HEK 293 cell lysates after which increasing amounts (0, 0.156, 1.56, and 7.8 mM) of the copper chelators tetrathiomolybdate (TM) and bathocuproinedisulfonic acid (BCS) and zinc chloride were added. XIAP mobility was subsequently determined by immunoblotting.

(D) BCS (15.6 mM) was added to hepatic tissue lysate from an affected cocker spaniel and subsequently probed for XIAP, COMMD1, and β-actin.

analysis of XIAP showed no significant changes in XIAP mRNA expression levels (A. Wilkinson and C.S.D., unpublished data), indicating that the reduction in XIAP occurs posttranscriptionally.

Because copper is a strong oxidant, we sought to determine whether the concentration of copper used in our studies induced cellular oxidation or was otherwise toxic to the cell. As shown in Figure 2C, incubation with copper induced neither reactive oxygen species (ROS) formation nor cytoxicity (Figure 2C). Furthermore, changes in the mobility or levels of XIAP were not observed after the addition of other divalent metals such as zinc or iron, or hydrogen peroxide, a source of ROS (Figure 2D). These findings support the notion that copper induces alterations in XIAP of a highly specific nature.

The Mobility Shift of XIAP Can Be Recapitulated In Vitro and Is Reversible

One potential explanation to account for the copper-induced alteration in the electrophoretic mobility of XIAP is that copper might be inducing the proteolysis of one or both termini. To test this possibility, an expression vector was constructed encoding a doubly tagged XIAP modified to incorporate both an amino-terminal FLAG tag and a carboxy-terminal HA tag in the same protein. This plasmid was transfected into 293 cells, which were either left untreated or treated with copper, and samples were examined by immunoblot separately with anti-FLAG and anti-HA antibodies. In the lysates from cells incubated with copper (Figure 3A), the faster-migrating band was again observed, similar to that seen with endogenous XIAP, and furthermore, this altered mobility was detected with both FLAG- and HA-specific antibodies. These data indicate that neither terminus of the molecule undergoes proteolytic cleavage to produce the electrophoretically distinct form of XIAP observed after copper treatment.

The lack of a proteolytic event suggested that the mobility shift might represent a conformational change in the protein triggered by copper. Therefore, we tested whether the mobility shift could be recapitulated in vitro by incubating whole-cell lysates from HEK 293 cells with increasing amounts of copper. This resulted in a graded mobility shift of XIAP (Figure 3B, top) that resembled the phenomenon observed when incubating 293 cells in media supplemented with copper. This mobility shift has been observed in all cell lysates examined to date, tested from a wide range of cell types, including Jurkat, FL5.12, Ht29, Caco-2, and HeLa cell lines, suggesting that the conformational change in XIAP observed after copper treatment is a widely occurring phenomenon.

The observation that copper treatment was able to induce a mobility shift of XIAP in whole-cell lysates raised the question of whether this was an intrinsic property of XIAP or required other cellular factors. To explore this question in more detail, the ability of purified XIAP to undergo a mobility shift was tested. As shown in Figure 3B (bottom), recombinant bacterially expressed XIAP demonstrated a mobility shift that was essentially identical to that seen in whole-cell lysates after incubation with increasing concentrations of copper. These findings suggest that the mobility shift is intrinsic to XIAP and does not require other cellular cofactors.

Previous studies have described the ability of IAPs to bind zinc (Clem et al., 2001; Hinds et al., 1999; Miller, 1999; Sun et al., 1999), raising the possibility that like zinc, copper may also bind directly to XIAP. In that case, the mobility shift could potentially be reversed by copper chelation. In order to test this hypothesis, copper was added to whole-cell extracts to induce the mobility shift of XIAP (as in Figure 3B), and then increasing amounts of two different copper chelators, tetrathiomolybdate (TM) and bathocuproinedisulfonic acid (BCS), were added. Copper chelation restored the slower-migrating form of

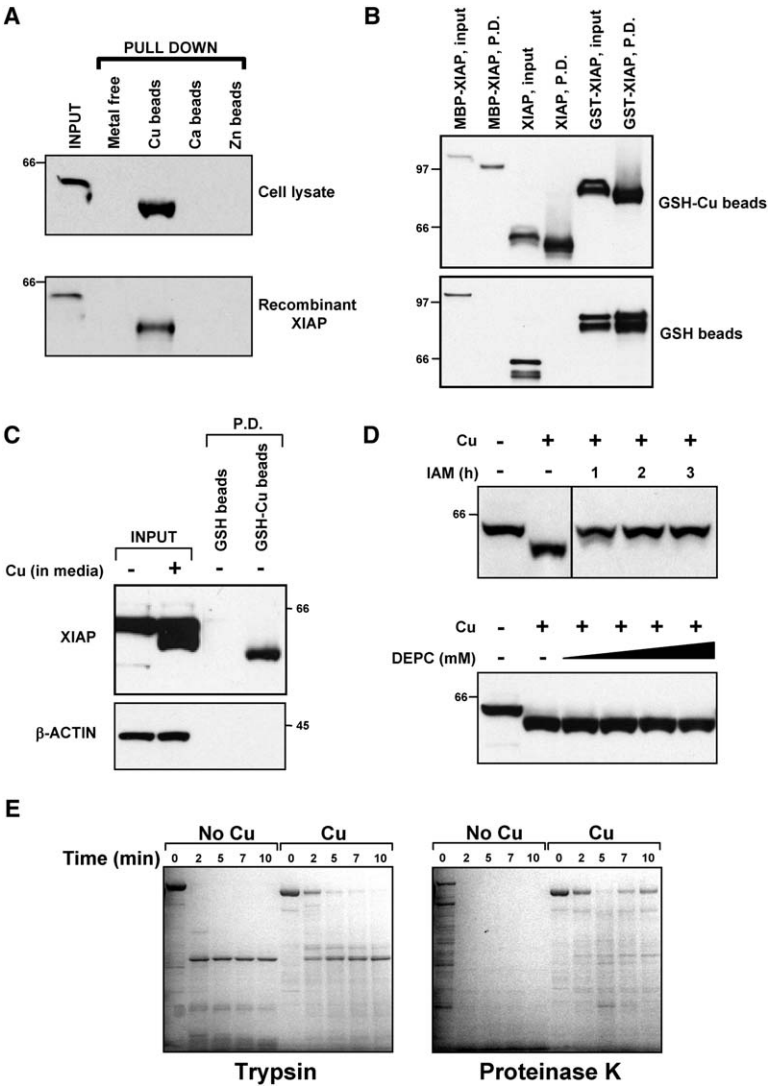

Figure 4. XIAP Binds Copper Directly and Undergoes a Conformational Change

(A) Metal-free and copper-, calcium-, and zinc-chelated beads were added to HEK 293 cell lysates and recombinant XIAP to ascertain which metals precipitate XIAP. Precipitated (pull-down) samples were subsequently probed for XIAP.

(B and C) Copper was loaded onto GSH beads and used to precipitate recombinant XIAP untagged and in fusion with MBP or GST (B) and similar precipitations were performed with cellular XIAP from HEK 293 cell lysates (C). XIAP and β-actin were detected by immunoblotting.

(D) Copper binds specifically to cysteine residues on XIAP. Copper binding to cellular XIAP (from HEK 293 lysates) was tested in the presence of iodocetamide (IAM) or diethyl pyrocarbonate (DEPC), which interfere with binding to cysteine and histidine residues, respectively. Protein was incubated on ice with IAM (31.2 mM) for 1, 2, or 3 hr (top) or with DEPC (0.5 mM, 1 mM, 2 mM, and 3 mM) for 3 hr. Copper sulfate (1.56 mM) was then added to induce the mobility shifted form of XIAP, which was detected by immunoblotting.

(E) Native and copper bound XIAP exhibit different patterns of digestion with trypsin and proteinase K. Native and copper bound XIAP were digested with trypsin and proteinase K for defined time points. The trypsin digests were then terminated by the addition of 5 mM phenylmethylsulfonyl fluoride (PMSF), and the proteinase K digests were terminated by boiling the samples in MBP elution buffer. The samples were then run on a denaturing polyacrylamide gel and the bands visualized with Coomassie staining technique.

XIAP (Figure 3C), indicating that the effect of copper on XIAP is a reversible change. In addition, increasing concentrations of zinc did not reverse the mobility shift, suggesting that there are distinct metal binding sites for copper and zinc. To investigate whether these results could be recapitulated in a cellular system, HEK 293 cells were cultured in media supplemented with copper, in the absence or presence of the cell-permeable copper chelator tetraethylenepentamine (TEPA). The presence of TEPA in the culture media similarly was sufficient to prevent the copper-induced mobility shift of XIAP (Figure S1B). Finally, the mobility shift observed in canine tissue described in Figure 1B was also reversed by the addition of copper chelators to the liver tissue lysate (Figure 3D). This finding strongly supports the idea that the altered mobility of XIAP as a consequence of copper overload in vivo is due to a similar process as that observed in cell culture.

Direct Binding of Copper to Cysteine Residues within XIAP Induces a Major Conformational Change in XIAP

The reversible nature of the mobility shift supported the notion that this event might be the result of direct copper binding by XIAP. To test this possibility, beads coupled with the metal chelator pentadentate (PDC) and in complex with various metals were used to precipitate protein from either whole-cell extracts or purified recombinant XIAP. As shown in Figure 4A, beads with immobilized copper, but not other metals, were found to efficiently precipitate recombinant XIAP (bottom) or endogenous XIAP from cell lysates (top). Interestingly, the precipitated XIAP migrated in the shifted position, consistent with the incorporation of copper into XIAP.

To independently confirm the observation that XIAP binds copper directly, we took advantage of the high affinity of glutathione (GSH) for copper. GSH Sepharose beads were either untreated or loaded with copper and then extensively washed to remove excess metal and subsequently tested for their ability to precipitate XIAP. Recombinant XIAP variants were used that were either untagged or contained amino-terminal affinity tags (maltose binding protein [MBP] or glutathione-S-transferase [GST]). As expected, native GSH Sepharose beads were only able to precipitate GST-XIAP, due to the high affinity of GST for GSH (Figure 4B, bottom). However, GSH beads in which copper had been adsorbed efficiently precipitated all recombinant proteins,

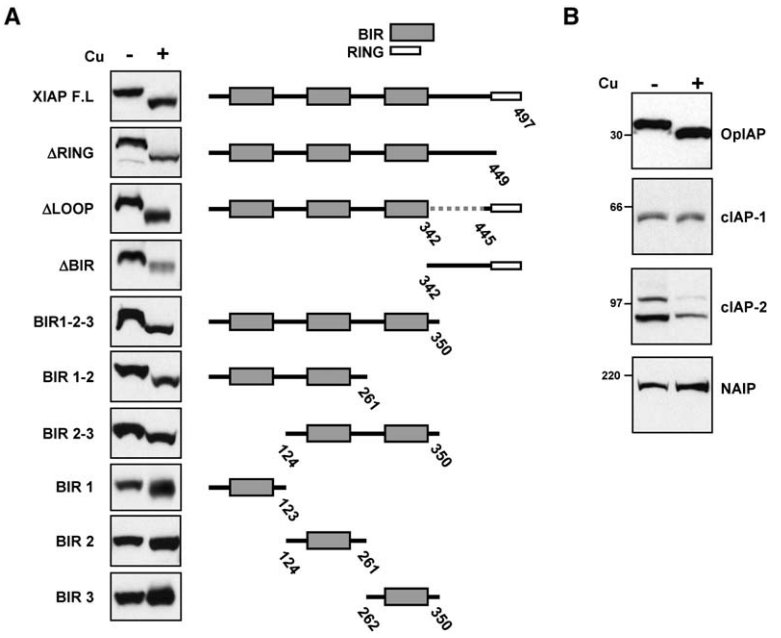

Figure 5. Copper Binding Is Mediated by Multiple Domains of XIAP and Is Shared by Other IAPs

(A) Multiple domains of XIAP can mediate the copper-induced shift. The indicated deletion mutants of XIAP were expressed in HEK 293 cells, which were subsequently lysed, and copper sulfate (1.56 mM) was added to the lysates. The mobility of XIAP was examined by immunoblotting.

(B) Other IAPs undergo a mobility shift on NuPAGE in the presence of copper. The indicated IAPs were transiently expressed in HEK 293 cells, copper sulfate (50 μM) was added to the media, and cell lysates were subsequently examined by immunoblotting using the relevant antibodies.

including those lacking a GST tag, confirming a strong and specific affinity of XIAP for copper. The recovered material migrated in the shifted position (Figure 4B, top), as seen also with the beads in which copper had been directly chelated (Figure 4A). A similar experiment performed with whole-cell lysates demonstrated that only copper-loaded GSH beads and not native GSH beads were capable of specifically precipitating XIAP, and the recovered protein migrated in the shifted position (Figure 4C). Taken together, these results indicate that XIAP directly binds to copper and undergoes a conformational change manifested by an alteration in its electrophoretic mobility.

The studies described above and in Figure 4 indicate a direct interaction between XIAP and copper. Because numerous previous reports have described critical roles for cysteine and histidine residues in copper binding (Adman, 1991), we tested the possibility that these residues may also be involved in the copper-XIAP interaction. Iodoacetamide (IAM) is an alkylating agent that avidly binds cysteine thiols and, thus, can neutralize the ability of cysteine residues to bind copper. We therefore compared the ability of XIAP to undergo a copper-induced mobility shift in whole-cell lysates, in the presence and absence of IAM. Significantly, IAM was sufficient to prevent the copper-mediated shift of XIAP (Figure 4D, top). Conversely, the addition of diethyl pyrocarbonate (DEPC), a histidine selective reagent, did not ablate the shift (Figure 4D, bottom). These results indicate that copper binds to cysteine, but not histidine residues within XIAP.

The altered mobility shift of XIAP observed after copper treatment, its reversibility by copper chelation, and the ability of XIAP to bind directly to copper are all suggestive of a conformational change induced by copper binding. To explore in more detail the possibility that XIAP undergoes a change in its conformation after binding to copper, we performed limited proteolytic digestion analysis of purified, recombinant XIAP in its free or copper-shifted form by using trypsin (Figure 4E, left) and proteinase K (Figure 4E, right). As shown in Figure 4E, the copper bound form of XIAP exhibits very different patterns of digestion to native XIAP, with both proteolytic enzymes over a 10 min period at 37ºC, strongly indicating that XIAP adopts a distinct conformation when bound to copper. The resistance of copper bound XIAP to proteases seen here reflects a conformational change in the protein and does not necessarily signify an altered susceptibility to degradation in vivo. The digestion patterns obtained with an unrelated control protein, bovine serum albumin, were identical in the presence and absence of copper with both proteinase K and trypsin (data not shown), indicating that their proteolytic activity was unaffected by the supplemental copper.

Copper Binding Is Mediated by Multiple Regions in XIAP

The iodoacetamide experiment shown in Figure 4D indicated that cysteine residues are required for XIAP to bind copper, prompting us to examine the domains within XIAP that might mediate copper binding. XIAP contains three BIR domains that are known to coordinate zinc, consistent with their similarities to zinc fingers (Hinds et al., 1999; Sun et al., 1999). These BIR domains are cysteine rich, and similarly, the carboxy-terminal RING finger domain present in XIAP is also rich in cysteine residues (Yang et al., 2000) and known to coordinate zinc. Therefore, the potential involvement of various domains of XIAP in copper binding was evaluated by testing the ability of truncated versions of the protein to undergo the mobility shift after addition of copper sulfate to the lysate. As shown in Figure 5A, the amino-terminal portion of XIAP containing the three BIR domains and lacking the RING finger domain was capable of undergoing a mobility shift as were the BIR1-2 and BIR2-3 constructs. In addition, a truncation mutant lacking all BIR domains and containing the RING finger domain

was also capable of undergoing the mobility shift. These results indicate that the BIR and RING domains of XIAP are all likely involved in copper binding, and these results are consistent with both the stepwise nature of the mobility shift observed in vitro (Figure 3B) and the known requirement of cysteine residues for copper binding to XIAP.

Copper Can Induce Mobility Shift of Other IAPs

Given the structural and functional similarities between various members of the IAP family, we investigated whether the electrophoretic mobility shift observed with XIAP could also be induced with other IAPs. To this end, HEK 293 cells were transfected with expression vectors for the IAPs indicated in Figure 5B, and the cells were then cultured in media supplemented with copper sulfate. Interestingly, this resulted in a mobility shift for the prototype baculoviral Op-IAP, suggesting that the copper binding properties of IAPs might be evolutionarily conserved. Although several other members of the IAP family did not appear to shift, treatment of cIAP-2-expressing cells with copper resulted in a redistribution of the doublet typically observed after overexpression of this gene into predominantly the faster-migrating species. These findings suggest that the ability of XIAP to bind copper is likely to be shared with several other IAP family members.

Copper Bound XIAP Exhibits Impaired Caspase-3 Inhibitory Activity and Renders Cells More Susceptible to Apoptotic Stimuli

Protection from apoptosis by XIAP relies primarily on its ability to inhibit caspases (Deveraux and Reed, 1999). Therefore, the ability of untreated and copper bound XIAP to inhibit caspase-3 was compared. Preliminary experiments revealed that even trace amounts of free copper abrogate caspase-3 activity (A.R.M. and C.S.D., unpublished data). We therefore devised a strategy to test the caspase inhibitory properties of copper bound XIAP in the absence of free copper. Recombinant GST or GST-XIAP were first adsorbed onto native or copper bound GSH Sepharose beads, which were subsequently washed to remove free copper. This allowed us to induce the copper bound form of XIAP but remove excess free copper from the system. In control samples, copper immobilized onto the GSH beads did not substantially affect the assay when compared to other negative controls such as native GSH beads either alone or bound to GST.

The enzymatic activity of recombinant caspase-3 was determined by its ability to cleave the fluorogenic substrate DEVD-AFC in the presence of increasing amounts of recombinant native or copper bound GST-XIAP. The caspase-3 activity in the presence of GST-XIAP was compared to that seen in the presence of equal amounts of GST to derive a percentage activity (Figure 6A). As expected, increasing amounts of GST-XIAP bound to native GSH Sepharose beads resulted in progressive inhibition of caspase-3 activity. However, GST-XIAP immobilized onto copper bound GSH beads no longer inhibited caspase-3. These results indicated that binding of copper by XIAP induces a loss of caspase inhibition, most likely due to the resulting conformational changes.

The effect of copper binding on the ability of XIAP to bind to caspase-3 was then investigated. Again,

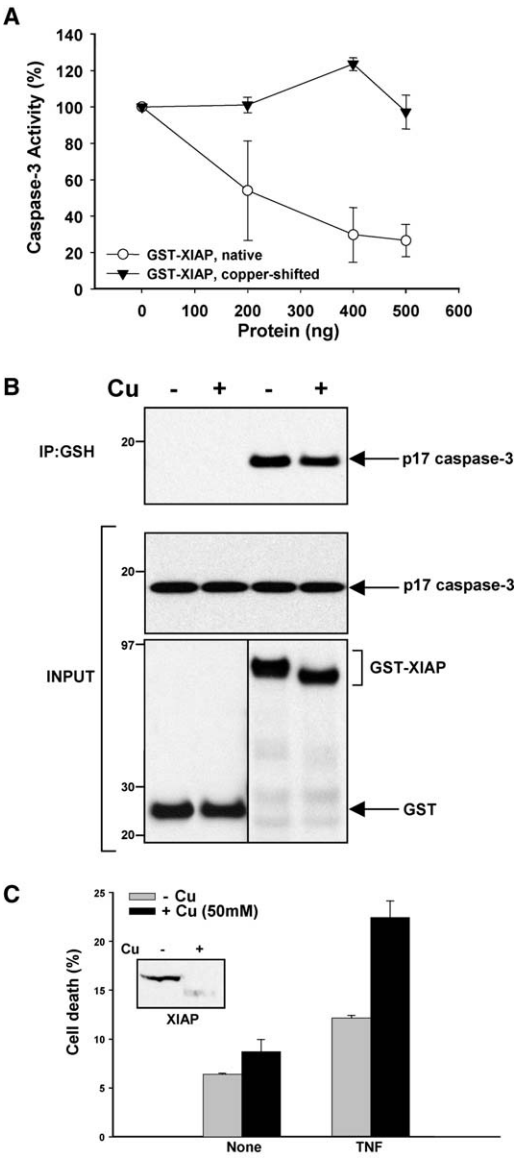

Figure 6. Copper Bound XIAP Does Not Inhibit Caspase 3 and Decreases the Threshold for Apoptosis

(A) Effects of the copper-induced mobility shift on the caspase-3 inhibitory activity of XIAP. Caspase-3 activity in the presence of copper-shifted GST-XIAP is compared to the native GST-XIAP control. Recombinant GST and GST-XIAP were mixed with glutathione beads, either untreated or after copper immobilization, the latter resulting in a copper-induced mobility shift. Caspase-3 was then added and its enzymatic activity determined by the cleavage of the fluorogenic substrate DEVD-AFC, and the activity in the presence of GST-XIAP was compared to the corresponding GST control to derive a percentage activity. The mean percentage activity and standard deviation of three samples per group are presented.

(B) The ability of native and copper bound GST-XIAP to bind caspase-3 was determined by coprecipitation and immunoblotting as indicated.

(C) Cells expressing the copper bound form of XIAP are more susceptible to apoptosis. HEK 293 cells were grown in the presence of additional copper sulfate (50 μM), and a combination of TNF (500 U) and CHX (60 μg/ml) was used to induce cell death. PI staining was used to identify dead cells by flow cytometry. The mean and standard deviation of three samples per group are shown.

recombinant GST or GST-XIAP was first bound to GSH Sepharose beads (either untreated or copper bound) and mixed with recombinant caspase-3. After precipitation and washing of the beads, the presence of caspase-3 was determined by immunoblotting (Figure 6B). Interestingly, the copper bound form of XIAP was still capable of binding caspase-3, despite its inability to inhibit caspase-3 enzymatic activity.

Based on the finding that copper bound XIAP is a poor inhibitor of caspase-3, we next investigated whether cells expressing the mobility-shifted form of XIAP were more susceptible to apoptotic stimuli. Cells were cultured in media supplemented with copper to induce the shifted form of XIAP (Figure 6C, inset) and then challenged with tumor necrosis factor (TNF) as a death stimulus. Although at this time-point, the dose of TNF used (500 U) resulted in minimal cell death in control cultures, cells expressing the mobility shifted form of XIAP were greatly sensitized to death (Figure 6C), and this occurred under conditions in which copper did not induce ROS formation (Figure 2C). Although the possibility cannot formally be excluded that copper can sensitize these cells to apoptosis through XIAP-independent pathways, these data suggest that at least one way to achieve this sensitization can be through the inactivation of XIAP.

Discussion

XIAP was originally identified as a mammalian homolog of the antiapoptotic protein Op-IAP and has since been found to bind and directly inhibit caspase-3, caspase-7, and caspase-9 (Chai et al., 2001; Deveraux and Reed, 1999; Riedl et al., 2001; Shiozaki et al., 2003). In addition to its role in regulating apoptosis, XIAP can also regulate intracellular copper levels by functioning as an E3 ubiquitin ligase for COMMD1, a factor involved in copper homeostasis. Consistent with this, tissues from *Xiap*-deficient mice contain elevated levels of COMMD1, as well as reduced levels of copper (Burstein et al., 2004). In light of this role for XIAP in copper homeostasis, we investigated the potential effects of copper on XIAP.

In this study, we demonstrate that in the presence of elevated copper, XIAP levels are markedly reduced both in inherited and acquired copper toxicosis disorders and that XIAP adopts an alternative conformation that has enhanced electrophoretic mobility in polyacrylamide gels, even under reducing, denaturing conditions. This conformational change is specifically induced by copper and occurs as a result of direct binding of the metal to multiple cysteine residues within XIAP. This finding is consistent with previous studies that have shown that other copper binding proteins such as ATP7B utilize cysteine residues to complex copper (Ralle et al., 2004). The BIR domains and the RING finger, which are all rich in cysteines, appear to be capable of binding copper, and the deletion studies performed here confirmed that multiple regions of XIAP can bind to copper. Additionally, other IAPs such as Op-IAP and cIAP-2 (Figure 5B) are also affected by intracellular copper accumulation, raising the possibility that the functions of IAPs in general might be affected under conditions of copper excess. Thus, copper overload may have a more profound effect on the apoptotic threshold beyond XIAP inactivation alone.

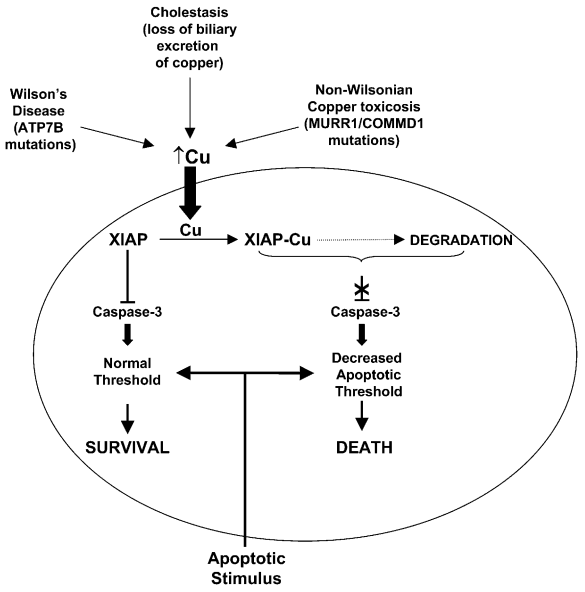

Figure 7. Overview of the Relationship between Copper, XIAP, and the Apoptotic Threshold

Intracellular copper accumulation results in a copper-induced conformational change of XIAP, thereby increasing its intracellular degradation and decreasing its ability to inhibit caspase-3. These changes result in a lowering of the apoptotic threshold and increased cell death in response to apoptotic stimuli.

In its copper bound state, XIAP is more susceptible to degradation than in its native form and is greatly impaired in its ability to inhibit caspase-3 despite the fact that there is no observable difference in its ability to bind the active p-17 caspase-3 fragment. Structural studies indicate that the interaction between XIAP and caspase-3 results in steric interference of the enzymatic site of caspase-3 by a domain in XIAP located just upstream of BIR2 (Riedl et al., 2001). The fact that the copper bound form of XIAP still binds caspase-3 but does not inhibit its activity implies that the mechanism of binding does not involve the active site of the enzyme. This finding is not surprising in light of the conformational change that XIAP undergoes when bound to copper, as demonstrated by the different digestion patterns seen with native and copper bound XIAP with proteinase K and trypsin. Importantly, cells expressing the mobility-shifted form of XIAP are more sensitive to apoptotic stimuli, and this is probably due to a decrease in XIAP levels in conjunction with the inability of the copper bound form of the protein to inhibit caspase-3.

These findings provide a provocative and unexpected link between cellular copper homeostasis and the control of apoptosis. Abnormal copper accumulation such as that resulting from Wilson's disease or chronic cholestasis is accompanied by cell death that has long been attributed to a direct toxic effect of copper. The data presented here suggest that in the setting of intracellular copper accumulation, XIAP undergoes a reversible conformational change that enhances its susceptibility to intracellular degradation and significantly decreases its ability to inhibit caspase-3. This results in a lowering of the apoptotic threshold of the cell and, in the presence of an apoptotic stimulus, leads to increased cell

death (Figure 7). Future studies will determine the extent to which this process contributes to the pathophysiology of copper toxicosis diseases.

This change in the biological properties of XIAP after copper binding is akin to that observed in other proteins that have a high affinity for copper. Amyloid-β-protein (Aβ) and prion protein (PrP^c) both avidly bind copper, and this interaction plays an important role in the eventual development of Alzheimer's disease (Bush et al., 2003) and transmissible spongiform encephalopathies, respectively (Millhauser, 2004). In the same way, the lowering of the apoptotic threshold in copper overload disorders may play a pivotal role in hastening cell death, resulting in the accelerated development of a disease phenotype. This raises the intriguing possibility that copper chelator therapy in Wilson's disease may be effective not only because excess copper is removed from the cell but also because the apoptotic threshold is normalized. Conversely, harnessing the effects of copper on XIAP may have practical applications in tumors, because it would be predicted that increases in copper may actually function synergistically with small molecule XIAP antagonists to sensitize cells to apoptosis.

Experimental Procedures

Plasmids

The plasmids pEBG-BIR1, pEBG-BIR2, pEBG-BIR3, pEBG-BIR1-2, pEBG-BIR2-3, and pEBG-BIR1-2-3 were generated by PCR using pEBB-XIAP as a template (boundaries are indicated in Figure 5). The plasmids pEBB-FLAG-XIAP-HA and pMAL-c2x-XIAP were derived by incorporating the XIAP coding sequence into the vectors pEBB-FLAG-HA and pMAL-c2x, respectively. The plasmids pEBG-ΔBIR, pEBG-ΔLOOP, pEBG-ΔRING and pEBG-XIAP, pcDNA3-OpIAP-HA, pcDNA3-Myc_6-cIAP-2, pcDNA3-Myc_6-NAIP, and pEBB-cIAP-1 have been described previously (Duckett et al., 1998; Liston et al., 1996).

Cell Culture, Transfection, and Hepatocyte Isolation

Human embryonic kidney 293 cells were cultured in DMEM supplemented with 10% FBS and 2 mM L-glutamine. A standard calcium phosphate transfection protocol (Duckett et al., 1997) was used to transfect 293 cells in all cases. *Atp7b*$^{tx\text{-}J}$ toxic milk mice (Coronado et al., 2001) and littermate controls were obtained from The Jackson Laboratory, Bar Harbor, ME. Hepatocytes were isolated by a modified in situ collagenase perfusion technique (Wan et al., 1995).

In Vitro Induction of Copper-Mediated XIAP Mobility Shift

Recombinant XIAP and GST-XIAP were prepared as previously described (Lewis et al., 2004). MBP-XIAP fusion protein was produced by following the manufacturer's instructions (NEB). The indicated concentrations of copper sulfate were added to whole-cell lysates, recombinant XIAP, and GST-XIAP on ice. After mixing, the samples were immediately resolved on denaturing NuPAGE 4%–12% polyacrylamide gradient gels as described below. In the stated experiments, after the addition of copper to the lysates as described above, increasing concentrations (0.156 mM, 1.56 mM, and 7.8 mM) of two different copper chelators, BCS (Sigma) and TM (Sigma), were added to the lysates and incubated on ice for 2 hr.

Precipitations Using Metal-Chelated Beads

Metal-free beads or ones with copper, zinc, or calcium immobilized onto a pentadentate chelator coupled to a quartz base matrix (PDC-SLQ free, Cu-PDC-SLQ, Zn-PDC-SLQ, and Ca-PDC-SLQ) were obtained from Affiland (Liege, Belgium). In addition, copper was also immobilized onto GSH by rotating equal volumes of GSH Sepharose beads (GE Healthcare) with copper sulfate (50 mM) at 4ºC for 1 hr. The beads were then washed five times (twice the bead bed volume) with EDTA-free 1% Triton lysis buffer (25 mM HEPES, 100 mM NaCl, 10% glycerol, and 1% Triton X-100) to remove excess unbound copper. The above beads were then used to precipitate recombinant XIAP (untagged or tagged with GST or MBP) and protein from HEK 293 cell lysates as indicated. Thirty microliters of beads was added to either recombinant protein or whole-cell lysates and rotated at 4ºC for 2 hr. The beads were then washed four times with EDTA-free 1% Triton lysis buffer, pelleted, and the precipitate resuspended in LDS loading buffer for immunoblotting.

Antibodies, Immunoblotting, and Immunoprecipitation

All tissue samples were lysed in Laemmli buffer (0.0625 mM Tris-HCl, 2% SDS, 10% glycerol, and 5% β-mercaptoethanol). Lysates from cultured cells were prepared with a Triton X-100 lysis buffer (25 mM HEPES, 100 mM NaCl, 10% glycerol, and 1% Triton X-100) supplemented with protease inhibitors. Protein samples were resolved by using 4%–12% gradient Novex Bis-Tris gels (Invitrogen), transferred to nitrocellulose membranes (Invitrogen), and blocked with 5% milk solution in TBS containing 0.05%–0.2% Tween-20. The membranes were incubated with the relevant primary antibodies followed by incubation with HRP-conjugated secondary antibodies (GE Healthcare). Antibody detection was performed by the Enhanced Chemiluminescence (ECL) Western blot analysis system (GE Healthcare). Antibodies against XIAP (Transduction Labs), GST (Santa Cruz), FLAG (Sigma), HA (Sigma), β-actin (Sigma), and COMMD1 (Burstein et al., 2005) were used.

Cell Viability Studies

Copper (50 μM) was added to HEK 293 cells as described above. After 48 hr, a combination of TNF (500 U; Roche) and CHX (60 μg/ml; Sigma) was used as the apoptotic stimulus. Alternatively, etoposide (30 μg/ml) alone was added in the absence of copper. After incubation for 16 hr, the cells were collected in 5 ml of PBS and washed once with 3 ml PBS. The samples were centrifuged at 200 × g for 5 min, the PBS was aspirated, and the cells were washed once more with 1 ml PBS. After further centrifugation at 200 × g for 5 min, the pelleted cells were resuspended in 0.5 ml PBS containing 2 μg/ml propidium iodide (PI) and incubated on ice for 10 min. PI-positive dead cells were then detected by flow cytometry.

Detection of Reactive Oxygen Species

The fluoroprobe 5-(and 6-)chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA, Invitrogen) was used to detect ROS production. Either copper (50 μM) or etoposide (30 μg/ml) was added to HEK 293 cells for 48 and 16 hr, respectively. Subsequently, CM-H_2DCFDA was added to the cells to a final concentration of 1 μM for 30 min, and the cells were then detached by using trypsin after being washed with 1 ml PBS. The samples were centrifuged at 200 × g for 5 min, the PBS was aspirated, and the cells were washed once more with 2 ml PBS. After further centrifugation at 200 × g for 5 min, the pelleted cells were resuspended in 0.5 ml PBS and ROS-positive cells were detected by flow cytometry.

Degradation Studies

HEK 293 culture media was supplemented with copper sulfate (50 μM) to induce the mobility shift of XIAP. After 48 hr, CHX (30 μg/ml) was added to the growth media to inhibit de novo protein synthesis, and the cells were then lysed at different time points as indicated. The samples were then prepared for immunoblot analysis, and XIAP and β-actin were identified by immunoblotting.

Protease Digestion

The copper bound form of the protein was generated by adding copper sulfate (1.56 mM) directly to recombinant MBP-XIAP. and binding of copper to the protein was confirmed by the presence of the mobility shift on the Coomassie blots. The proteolytic sensitivity of both native and copper bound MBP-XIAP was determined by carrying out timed digests with trypsin and proteinase K (Jakob et al., 2000). The samples were separated by SDS-PAGE and the protein bands visualized by colloidal Coomassie staining (Invitrogen).

Supplemental Data

Supplemental Data include one figure and can be found with this article online at http://www.molecule.org/cgi/content/full/21/6/775/DC1/.

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